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Gas Chromatographic/Mass Spectrometric Assay for Profiling the Enantiomers of 3,4-Methylenedioxymethamphetamine and its Chiral Metabolites using Positive Chemical Ionization Ion Trap Mass Spectrometry

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A qualitative GC/MS profile was obtained and its mass spectrometric features characterized for the analysis of the enantiomers of (*RS*)-3,4-methylenedioxymethamphetamine (MDMA) and its metabolites (*RS*)-3,4-methylenedioxyamphetamine (MDA), (*RS*)-4-hydroxy-3-methoxymethamphetamine (HMMA) and (*RS*)-4-hydroxy-3-methoxyamphetamine (HMA). A chiral derivatization method was selected to obtain the diastereomers required for the separation of the respective enantiomers with a non-chiral GC stationary phase. The selected derivatization consisted of a reaction with *N*-heptafluorobutyryl-(*S*)-prolyl chloride combined with a consecutive reaction with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, resulting in *N*-[heptafluorobutyryl-(*S*)-prolyl]-*O*-trimethylsilyl derivatives. Detection was carried out with electron ionization and positive chemical ionization (PCI) ion trap mass spectrometry. Mass spectra of the derivatives of reference standards of the compounds of interest obtained with PCI demonstrated that this method simultaneously induces proton and charge-transfer reactions in the ion trap. The advantage is that high mass information is provided while some fragmentation remains to elucidate structural details. Subsequently, in three urine samples obtained from different and unrelated MDMA intoxications, the enantiomers of MDMA and MDA were identified. In some urine samples also HMMA and/or HMA were found. In addition to these compounds, an unexpected compound and/or additional chiral metabolite, *N*-hydroxy-(*RS*)-3,4-methylenedioxyamphetamine, was identified in two out of three urine samples. Preliminary results also indicated an enantioselective metabolism in the *N*-demethylation pathway for MDMA in humans.

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INTRODUCTION

The importance of enantiometric profiling in pharmacodynamics and toxicology in general[1] and in 3,4-methylenedioxymethamphetamine (MDMA) studies in particular[2,3] is well established. MDMA, more commonly known as 'Ecstasy' or 'XTC,' is an amphetamine analogue (Fig. 1), which is a common recreational drug of abuse in Europe. It has the reputation of being a mild intoxicant, although serious neurotoxic effects in rats

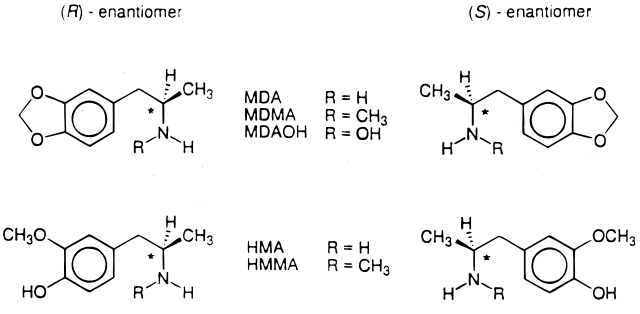

Figure 1. Molecular structures of the enantiomers of MDMA, some of its analogues and/or its chiral metabolites. Asterisks indicate a chiral center. MDMA = 3,4-methylenedioxymethamphetamine; MDA = 3,4-methylenedioxyamphetamine; MDAOH = *N* - hydroxy - 3 , 4 - methylenedioxyamphetamine; HMMA = 4-hydroxy-3-methoxymethamphetamine; HMA = 4-hydroxy-3-methoxyamphetamine.

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have been described.[4,5] In humans several complications after MDMA use have been reported.[6,7] Also sold under the names of 'Ecstasy' or 'XTC' are tablets containing the 3,4-methylenedioxy analogues 3,4-methylenedioxyamphetamine (MDA) and *N*-hydroxy-3,4-methylenedioxyamphetamine (MDAOH) (Fig 1).[8]

Illicitly synthesized MDMA usually is a racemic mixture. The two enantiomers of MDMA, however, show different pharmacodynamic properties. In animal models, (*S*)-MDMA is a more potent psychomimetic agent than the (*R*)-enantiomer and reportedly only the (*S*)-enantiomer is neurotoxic.[4] The major chiral metabolites, which in addition to the parent compound have been found in urine of animals and humans after deconjugation, are MDA, 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA)[9–12] (Fig. 1). The metabolism in animal models is enantioselective,[2] resulting in different *R*/*S* ratios of MDA and HMA (*R*/*S* ratio <1) compared with MDMA and HMMA (*R*/*S* ratio >1). For humans, such data are not yet available.

The goal of the present study was to develop a gas chromatographic/mass spectrometric (GC/MS) assay for the enantiomeric profiling of MDMA and its chiral metabolites and to characterize its mass spectrometric features. Because suitable and stable chiral stationary phases for GC are not readily commercially available, chiral derivatization methods are being recommended for the assay of enantiomers in MDMA studies.[2] In this way, enantiomers are converted into diastereomers which can be separated with a non-chiral GC stationary phase. Recently reported chiral derivatization methods for MDMA and/or MDA involved reaction with *N*-trifluoroacetyl-(*S*)-prolyl chloride,[3,13,14] *N*-pentafluoropropionyl-(*S*)-prolyl chloride[13] or *N*-heptafluorobutyryl-(*S*)-prolyl chloride,[2,13,14] resulting in *N*-[trifluoroacetyl-(*S*)-prolyl] (=*N*-(*S*)-TFAP), *N*-[pentafluoropropionyl-(*S*)-prolyl] (=*N*-(*S*)-PFPP) and *N*-[heptafluorobutyryl-(*S*)-prolyl] (=*N*-(*S*)-HFBP) derivatives, respectively. For the metabolites HMMA and HMA with additional hydroxy groups to be derivatized, a reaction with *N*-heptafluorobutyryl-(*S*)-prolyl chloride followed by a reaction with *N*-methylbis(trifluoroacetamide) was reported to be useful.[2] This reaction results in *N*-[heptafluorobutyryl-(*S*)-prolyl]-*O*-trifluoroacetyl (*N*-(*S*)-HFBP-*O*-TFA) derivatives. In our study, the second step in this chiral derivatization method for the metabolites HMMA and HMA was carried out with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, resulting in *O*-trimethylsilyl (*O*-TMS) derivatives, instead of *O*-TFA derivatives.

In addition to the chiral derivatization, the usefulness of ion trap mass spectrometry and the preliminary results of the enantiomeric profiles of MDMA and its chiral metabolites in human urine are described.

EXPERIMENTAL

Chemicals and reagents

(*RS*)-MDMA was a generous gift from Dr H. Huizer (Forensic Laboratory, Rijswijk, The Netherlands). (*S*)-Amphetamine, (*RS*)-methamphetamine and (*RS*)-propylhexedrine were kindly provided by Professor Dr J. M. van Rossum (Catholic University of Nijmegen, The Netherlands). A mixture of β-glucuronidase and arylsulfatase from *Helix pomatia* was obtained from Serva (Heidelberg, Germany). Anhydrous ammonium acetate (>98%), concentrated hydrochloric acid (37%, w/v), deuterotrichloromethane ($CDCl_3$) (>99%), dichloromethane (>99%), diethyl ether (>99%), ethyl acetate (>99.5%), iron(III) chloride (99%), formic acid (>98%), hexadeuterodimethyl sulfoxide (DMSO-d_6) (>99.8%), hydrogen peroxide (30% in water), propan-2-ol (>99%), lithium aluminum hydride (97%), anhydrous magnesium sulfate (>70% water free), methanol (99.8%), picric acid (>99.8%), potassium hydroxide (>85%), sodium chloride (>99.5%), sodium hydroxide (>99%), sulfuric acid (96%) and thionyl chloride were purchased from Merck Nederland (Amsterdam, The Netherlands). Iron powder (325 mesh, 97%), nitroethane (96%), safrole (97%), tetrahydrofuran (>99%), tetramethylsilane and vanillin (99%) were purchased from Acros Chimica (Geel, Belgium). Heptafluorobutyryl anhydride, (*S*)-proline (>99%) and sodium cyanoborohydride (95%) were obtained from Aldrich Chemie (Axel, The Netherlands). Tetrachloromethane (CCl_4) (>99%) was purchased from J. T. Baker (Deventer, The Netherlands). Methylamine hydrochloride (>98%) and deuterotrifluoroacetic acid (TFA-*d*) were purchased from Fluka (Buchs, Switzerland). *N*-Methylbis(trifluoroacetamide) (MBTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Macherey–Nagel (Düren, Germany). Trifluoroacetic anhydride (TFAA) was obtained from Pierce Europe (Oud-Beijerland, The Netherlands). Hydroxylamine hydrochloride (99%) was obtained from Sigma (St Louis, MO, USA). Silica (dry-column grade, activity III, 30 mm) for chromatography was obtained from Woelm (Eschwege, Germany). If not specified, the reagents and chemicals were of analytical grade.

Synthesis of reference standards of metabolites

The reference standards of metabolites and their starting materials were synthesized according to published synthetic routes and were characterized by proton nuclear magnetic resonance (^{1}H NMR) spectrometry and electron ionization (EI) GC/MS and, if specified, also by ^{13}C NMR spectrometry. ^{1}H NMR (90 MHz) was carried out with a Varian EM 390 instrument (Varian, Palo Alto, CA, USA) with tetramethylsilane as internal standard. ^{1}H NMR (300.07 MHz) and ^{13}C NMR (75.5 MHz) spectra of end products were recorded with a Gemini-300 BB instrument (Varian). In ^{1}H spectra the $CHCl_3$ line was set at 7.26 ppm and in ^{13}C spectra the central $CDCl_3$ signal was set at 76.9 ppm. GC/MS was performed with a Hewlett-Packard GC 5890 Series II instrument equipped with a CP-Sil 8 CB low-bleed MS fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm) (Chrompack, Middelburg, The Netherlands) coupled to a Hewlett-Packard Model 5972 mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). Mass spectral data were recorded from *m*/*z* 50 to 650 and are reported as normalized to the

base peak. Prior to GC/MS and if specified, the synthesized compounds were converted into trifluoroacetyl (TEA) derivatives. Ethyl acetate (50 μl) and 50 μl of TFAA were added to dry residues and the mixture was heated for 20 min at 60 °C. Excess of organic solvent and TFAA were removed by evaporation and the residue was dissolved in 100 μl of ethyl acetate. A 1 μl aliquot was injected into the GC/MS instrument. Melting points were determined in capillary tubes and are uncorrected.

Starting materials (**I–III**) for the synthesis of the reference standards were produced in several batches.

Piperonyl methyl ketone (I). Safrole was isomerized to isosafrole[15] by heating with 2.5 molar equivalents of potassium hydroxide for 6.5 h at 120 °C. After cooling to room temperature, the clear golden yellow solution was decanted (average yield >90%). The crude product was used in the next step as soon as possible, because isosafrole polymerizes slowly in the presence of moisture. Piperonyl methyl ketone was obtained by hydrogen peroxide oxidation of crude isosafrole followed by an acid-catalyzed rearrangement.[16] Distillation under reduced pressure yielded a clear yellow product (average yield 40%). ^{1}H NMR (90 Hz; CCl_4): δ 1.99 (s, 3 H, CH_3), 3.47 (s, 2 H, CH_2CO), 5.88 (s, 2 H OCH_2O), 6.49–6.72 (m, 3 H, aromatic). GC/MS: *m/z*, $M^{+\cdot}$ 178(26), 135(100), 77(23).

β-Nitroisoeugenol (II). Condensation of vanillin with nitroethane was carried out by the Knoevenagel condensation.[17,18] β-Nitroisoeugenol was obtained as yellow crystals, filtered off and air dried (average yield >55%). ^{1}H NMR (90 Hz; $CDCl_3$): δ 2.48 (s, 3 H, CCH_3), 3.95 (s, 3 H, OCH_3), 5.91 (bs, 1 H, OH), 6.85–7.17 (m, 3 H, aromatic), 8.08 (s, 1 H, CH). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 305(100), 258(52), 192(22), 178(16), 162(32), 161(49), 151(19), 147(35), 139(12), 131(36), 119(14), 115(66), 105(17), 103(34), 102(13), 91(27), 89(18), 79(18), 77(32), 69(68), 65(22), 63(15).

Vanillyl methyl ketone (III). β-Nitroisoeugenol was reduced with iron(III) chloride, iron powder and concentrated hydrochloric acid.[19] The resulting dark brown oil was purified by 'dry flash' column chromatography.[20] The product was eluted from a silica gel column with toluene–diethyl ether (1:2) (yield 68%). ^{1}H NMR (90 Hz; $CDCl_3$): δ 2.12 (s, 3 H, $COCH_3$), 3.64 (s, 2 H, CH_2), 3.90 (s, 3 H, OCH_3), 5.95 (bs, 1 H, OH), 6.66–7.02 (m, 3 H, aromatic). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 276(100), 233(75), 205(16), 137(16), 107(15), 106(10), 105(16), 77(16), 69(32), 65(13).

***(RS)*-MDA.** (*RS*)-MDA (1-(3,4-methylenedioxyphenyl)-2-aminopropane) was prepared from **I** by reductive amination with sodium cyanoborohydride.[21,22] To a solution of 17.7 g of anhydrous ammonium acetate in 60 ml of methanol were added 4.2 g of **I** followed by 1.7 g of sodium cyanoborohydride. The solution was stirred at ambient temperature for 72 h and kept at pH 6–7 by gradual addition of concentrated hydrochloric acid. After adding 100 ml of demineralized water, the pH was brought to 2 with concentrated hydrochloric acid. Methanol was removed *in-vacuo*. The aqueous solution was extracted with two consecutive portions of 50 ml of dichoromethane, brought to pH 10 with solid sodium hydroxide, saturated with sodium chloride and extracted three times with 50 ml portions of dichoromethane. The combined extracts were dried (magnesium sulfate) and the solvent was removed *in vacuo*. The resulting oil was dissolved in propan-2-ol and acidified with concentrated hydrochloric acid. A white solid substance crystallized spontaneously, was filtered off and washed first with propan-2-ol–diethyl ether (1:1) and then with diethyl ether. The product was isolated as (*RS*)-MDA hydrochloride (m.p. 187–188 °C, lit.[21] 187–188 °C; yield 31%). ^{1}H NMR (300.07 Hz; $CDCl_3$–TFA-*d* (6:1; v/v)): δ 1.45 (d, 3 H, J = 6.6 Hz, CCH_3), 2.86 (dd, 1 H, J_{AB} = 14.5 Hz, J_{BX} = 8.2 Hz, H-1), 2.95 (dd, 1 H, J_{AB} = 14.5 Hz, J_{AX} = 6.3 Hz, H-1), 3.68 (m, 1 H, CHN), 5.96 (s, 2 H, OCH_2O), 6.67 (dd, 1 H, J_o = 7.8 Hz, J_m = 1.8 Hz, H-6′), 6.69 (d, 1 H, J_m = 1.8 Hz, H-2′), 6.79 (d, 1 H, J_o = 7.8 Hz, H-5′), 6.9 (bs, 3 H, NH_3^+). ^{13}C NMR (75.5 Hz; $CDCl_3$–TFA-*d* (6:1; v/v)): δ 18.0 (CH_3), 40.3 (CH_2), 51.2 (CH), 101.2 (CH_2O), 109.0 (C-2′), 109.2 (C-5′), 122.5 (C-6′), 127.8 (C-1′), 147.3 (C-4′), 148.3 (C-3′). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 275(15), 162(38), 135(100), 77(16), 69(11).

***(RS)*-HMMA.** (*RS*)-HMMA (1-(3-methoxy-4-hydroxyphenyl)-2-methylaminopropane) was prepared from **III** by reductive amination with sodium cyanoborohydride.[21,22] To a solution of 3.0 g of methylamine hydrochloride in 20 ml of methanol were added 2.3 g of **III** followed by 1.0 g of sodium cyanoborohydride. The solution was treated as in the reductive amination of (*RS*)-MDA, except that the extraction was done with ethyl acetate instead of dichloromethane. Light brown crystals were obtained, which were filtered off and washed first with propan-2-ol–diethyl ether (1:1) and then with diethyl ether. The product was isolated as (*RS*)-HMMA hydrochloride (m.p. 181–189 °C; yield 12%). ^{1}H NMR (300.07 Hz; $CDCl_3$–TFA-*d* (6:1; v/v)): δ 1.41 (d, 3 H, J = 6.6 Hz, CCH_3), 2.80 (m, 3 H, NCH_3), 2.88 (dd, 1 H, J_{AB} = 14.2 Hz, J_{BX} = 7.2 Hz, H-1), 3.00 (dd, 1 H, J_{AB} = 14.2 Hz, J_{AX} = 7.2 Hz, H-1), 3.53 (m, 1 H, CH), 3.88 (s, 3 H, OCH_3), 6.70 (dd, 1 H, J_o = 8.1 Hz, J_m = 1.8 Hz, H-6′), 6.73 (d, 1 H, J_m = 1.8 Hz, H-2′), 6.90 (d, 1 H, J_o = 8.1 Hz, H-5′), 7.10 (bs, 2 H, NH_2^+), 11.38 (s, 1 H, OH). ^{13}C NMR (75.5 Hz; $CDCl_3$–TFA-*d* (6:1; v/v)): δ 15.3 (CH_3), 30.8 (NCH_3), 39.4 (CH_2), 55.9 (OCH_3), 58.2 (CH), 112.0 (C-5′), 115.2 (C-2′), 122.0 (C-6′), 126.9 (C-1′), 144.1 (C-4′), 147.1 (C-3′). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 387(<1), 260(24), 154(100), 110(24), 69(14).

***(RS)*-HMA.** (*RS*)-HMA (1-(3-methoxy-4-hydroxyphenyl)-2-aminopropane) was prepared from **II** by reduction with lithium aluminium hydride essentially according to Ramirez and Burger.[17] Instead of the Soxhlet extraction technique, 7.0 g of **II** were dissolved in 50 ml of dry tetrahydrofuran and the solution obtained was added dropwise to a well stirred mixture of 2.5 g of lithium aluminium hydride and 200 ml of dry diethyl ether. The dark red picrate obtained was dissolved in propan-2-ol and acidified with concentrated hydrochloric acid. A brown solid crystallized spontaneously and was filtered off and washed first with propan-2-ol–diethyl ether

(1:1) and then with diethyl ether. The product was isolated as (*RS*)-HMA hydrochloride (m.p. 244–246 °C; yield 9%). ^{1}H NMR (300.07 Hz; $CDCl_3$–TFA-*d* (3:1; v/v)): δ 1.46 (d, 3 H, $J = 6.6$ Hz, CCH_3), 2.89 (dd, 1 H, $J_{AB} = 14.5$ Hz, $J_{BX} = 8.4$ Hz, H-1), 3.00 (dd, 1 H, $J_{AB} = 14.5$ Hz, $J_{AX} = 6.2$ Hz, H-1), 3.73 (m, 1 H, CH), 3.90 (s, 3 H, OCH_3), 6.73 (dd, 1 H, $J_o = 7.9$ Hz, $J_m = 1.8$ Hz, H-6′), 6.78 (d, 1 H, $J_m = 1.8$ Hz, H-2′), 6.92 (d, 1 H, $J_o = 7.9$ Hz, H-5′), 11.23 (s, 3 H, NH_3^+), 11.23 (s, 1 H, OH). ^{13}C NMR (75.5 Hz; $CDCl_3$–TFA-*d* (3:1, v/v)): δ 18.0 (CH_3), 40.3 (CH_2), 51.2 (CH), 56.0 (OCH_3), 112.2 (C-5′), 115.4 (C-2′), 122.2 (C-6′), 127.5 (C-1′), 143.8 (C-4′), 147.3 (C-3′). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 373(3), 260(55), 233(12), 140(100), 69(21).

(*RS*)-MDAOH. (*RS*)-MDAOH (1-(3,4-methylenedioxyphenyl)-2-hydroxylaminopropane) was prepared from **I** by reductive amination with sodium cyanoborohydride.[21,22] To a solution of 14.8 g of hydroxylamine hydrochloride in 120 ml of methanol were added 3.6 g of **I** followed by 1.0 g of sodium cyanoborohydride. The solution was treated as in the reductive amination of (*RS*)-MDA. A white solid crystallized spontaneously and was filtered off and washed first with propan-2-ol–diethyl ether (1:1) and then with diethyl ether. The product was isolated as (*RS*)-MDAOH hydrochloride (m.p. 148–149 °C, lit.[21] 149–150 °C; yield 17%). ^{1}H NMR (300.07 Hz; $CDCl_3$–DMSO-d_6 (500:1, v/v)): δ 0.89 (d, 3 H, $J = 6.0$ Hz, CCH_3), 2.25 (dd, 1 H, $J_{AB} = 13.0$ Hz, $J_{BX} = 11$ Hz, H-1), 2.96 (dd, 1 H, $J_{AB} = 13.0$ Hz, $J_{AX} = 1.6$ Hz, H-1), 3.15 (m, 1 H, CHN), 5.58 (s, 2 H, OCH_2O), 6.08 (dd, 1 H, $J_o = 7.8$ Hz, $J_m = 1.8$ Hz, H-6′), 6.35 (d, 1 H, $J_m = 1.8$ Hz, H-2′), 6.34 (d, 1 H, $J_o = 7.8$ Hz, H-5′), 11.12 (s, 2 H, NH_2^+), 10.2 (bs, 1 H, NOH). ^{13}C NMR (75.5 Hz; $CDCl_3$): δ 12.8 (CH_3), 35.5 (CH_2), 57.9 (CH), 100.0 (CH_2O), 107.4 (C-2′), 108.6 (C-5′), 121.5 (C-6′), 128.9 (C-1′), 145.5 (C-4′), 146.8 (C-3′). GC/MS (TFA derivative): *m/z*, $M^{+\cdot}$ 291(6), 162(31), 135(100), 105(5), 77(15), 69(11).

Synthesis of (*S*)-HFBPCl

N-Heptafluorobutyryl-(*S*)-prolyl chloride ((*S*)-HFBPCl) was synthesized according to Lim *et al.*[23] The optical purity of (*S*)-HFBPCl was checked by GC/MS analysis of the derivatives of (*S*)-amphetamine, (*RS*)-methamphetamine and (*RS*)-MDMA. The reagent (*S*)-HFBPCl was stored under nitrogen at −20 °C as a 0.02 M solution in toluene and was stable for at least 3 months.

Urine sample collection

Urine samples were collected from intoxicated persons and delivered to the Department of Human Toxicology for analysis.

Isolation of MDMA and metabolites from urine

Apart from three modifications, the enzymatic hydrolysis of the conjugated metabolites and the extraction of MDMA and non- and deconjugated metabolites from urine samples were carried out according to Lim *et al.*[2] Instead of β-glucuronidase, a mixture of β-glucuronidase and arylsulfatase from *Helix pomatia* was used for enzymatic hydrolysis. (*RS*)-Propylhexedrine was used as an internal standard. The extraction was carried out with a mixture of dichloromethane and propanol (3:1, v/v). The organic solvent was removed in a gentle stream of nitrogen at 40 °C.

N-(*S*)-HFBP-*O*-TFA and -*O*-TMS derivatization

The *N*-(*S*)-HFBP-*O*-TFA derivatization was carried out according to Lim *et al.*[2] with the following modifications. To the dry residue after extraction, 0.5 ml of borate buffer (pH 9.0) was added. The solution was vortex mixed for 20 s and cooled in an ice-bath for 10 min. A 40 µl volume of (*S*)-HFBPCl reagent was added and the solution was again vortex mixed for 20 s and placed in an ice-bath for 20 min. A 200 µl volume of toluene was added, the solution was vortex mixed for 20 s and the phases were separated by centrifugation. The samples were stored at −20 °C for 30 min and the organic layer was decanted from the frozen aqueous phase into a clean tube. The solvent was removed in a gentle stream of nitrogen at 60 °C and the residues were dried under reduced pressure over phosphorous pentoxide and potassium hydroxide for at least 1 h. To the dry residue 50 µl of MSTFA were added. The solution was vortex mixed for 20 s and kept at 80 °C for 40 min. Excess reagent was removed in a gentle stream of nitrogen at 60 °C and the residue was dissolved in 200 µl of toluene. A 1 µl aliquot was analyzed by GC/MS.

GC/MS analysis of enantiomers

The GC/MS analyses were carried out with an Saturn II ion trap (Varian). The GC was provided with a septum-equipped programmable injector and a DB-5MS fused-silica capillary column (15 m × 0.25 mm i.d., film thickness 0.25 µm) (J&W Scientific, Folsom, CA, USA). The injector temperature program was as follows: the initial temperature (110 °C) was maintained for 1.5 min, then the temperature ramped to 280 °C at 100 °C min^{-1} and maintained there for 1 min. The oven temperature program was as follows: the initial temperature was maintained at 105 °C for 7.5 min, raised to 200 °C at 10 °C min^{-1}, maintained there for 10 min and then raised to 280 °C at 20 °C min^{-1}. The GC/MS analysis was performed either in the EI mode or in the positive chemical ionization (PCI) mode with isobutane (UCAR Union Carbide, Nieuw-Vennep, The Netherlands) as the reactant gas. All parameters were set according to the manufacturer's manual. Ratios of enantiomers were calculated as the peak height ratios of the protonated molecules in the PCI mode.

Statistical analysis

Statistical significance was assessed according to a Student's *t*-test.

RESULTS AND DISCUSSION

Selection of chiral derivatization

Because *N*-(*S*)-HFBP derivatives of compounds in general[14] and of MDMA in particular[13] are known to result in better chromatographic resolution and shorter retention times than *N*-(*S*)-TFAP and *N*-(*S*)-PFPP derivatives, it was decided to choose the *N*-(*S*)-HFBP derivatization for enantiomeric separation. An additional advantage is that the derivatization with (*S*)-HFBPCl can be carried out in aqueous solution. In our study, MSTFA was used in the second step of the chiral derivatization method necessary for the analysis of the metabolites HMMA and HMA, resulting in *O*-trimethylsilyl (*O*-TMS) derivatives (Fig. 2). Lim *et al.*[2] used MBTFA for the second step in order to produce *O*-TFA derivatives. However, in our hands the *N*-(*S*)-HFBP-*O*-TMS derivatization method proved to be more reproducible and to yield higher signals than the *N*-(*S*)-HFBP-*O*-TFA method (data not shown). The *N*-(*S*)-HFBP-*O*-TMS derivatization method was therefore chosen for the chiral GC/MS assay in this study. Probably *N*-(*S*)-HFBP-*O*-TFA derivatives are more useful for the analysis in the negative ionization mode, as reported by Lim *et al.*[2] In our study, only positive ions were analyzed.

Assessment of the optical purity of the (*S*)-HFBPCl reagent by GC/MS analysis of the (*S*)-amphetamine and (*RS*)-methamphetamine derivatives showed that racemization may occur after storing the reagent for more than 3 months. Racemization results in undesired (*N*-(*RS*)-HFBP derivatives. Similar problems were reported previously.[23] Storage of small amounts of (*S*)-HFBPCl reagent at a relatively low stock concentration under nitrogen, and therefore minimizing exposure to air during handling, extended the storage life.

Selection of ionization mode

In Table 1 the mass spectrometric data obtained with the EI mode are shown and in Schemes 1 and 2 the fragmentation mechanisms of some characteristic EI fragments are given.

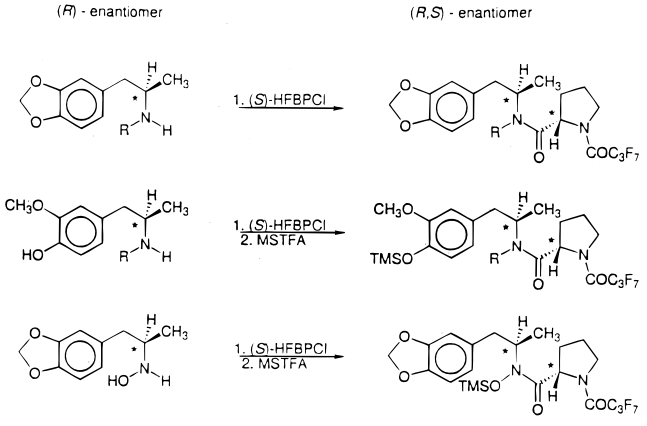
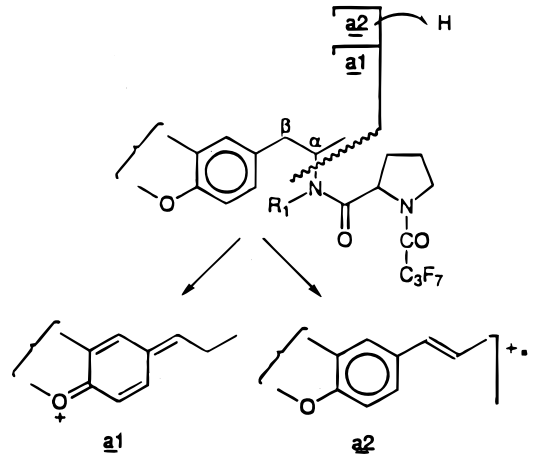
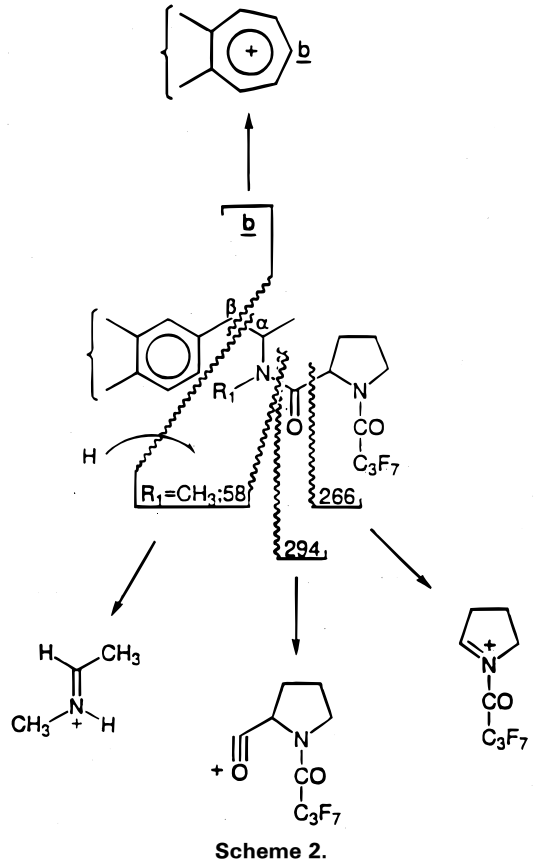


Figure 2. Formation of the *N*-heptafluorobutyryl-(*S*)-prolyl-*O*-trimethylsilyl derivatives of the (*R*)-enantiomers of the compounds studied. Asterisks indicate a chiral center. (*S*)-HFBPCl = *N*-heptafluorobutyryl-(*S*)-prolyl chloride; MSTFA = *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.



Scheme 1.

The EI spectra are dominated by the fragment at *m/z* 266, which is characteristic for *N*-HFBP derivatives, but not for the compound concerned (Scheme 2). Also typical for the *N*-HFBP derivative is the fragment at *m/z* 294 (Scheme 2). In the EI spectra most of the *m/z* values of the other fragment ions are smaller and their abundances lower than those at *m/z* 266 and although some of them are characteristic (fragments a_1, a_2, b and $R_1NH{=}CHCH_3^+$), others are not (fragments TMS^+ and CF_3^+). The characteristic fragments a_1, a_2 and b result from cleavage of the C_α—N bond (Scheme 1) and the C_β—C_α bond (Scheme 2), respectively. Except for MDA and HMA, the abundances of $M^{+\cdot}$ or combinations of $M^{+\cdot}$ with the loss an $F^\cdot$ radical are not significant. Significant loss of a $CH_3^\cdot$ radical was



Scheme 2.

Table 1. Partial mass spectrometric data[a] for *N*-HFBP-*O*-TMS derivatives of reference standards of MDMA and its chiral metabolites using the electron ionization mode

			m/z values of characteristic fragments (normalized on the base peak)[d]										
Compound[b]	Enantiomer No.[c]	M_r	$M^{+\cdot}$	$[M-CH_3]^+$	$[M-F]^+$	$C_3F_7CONC_4H_7CO^+$	$C_3F_7CONC_4H_7^+$	a_1	a_2	b	TMS^+	CF_3^+	$R_1NH{=}CHCH_3^+$
MDMA	1	486	486 (<1)	n.o.	467 (1)	294 (<1)	266 (100)	163 (12)	162 (56)	135 (14)	n.o.	69 (14)	58 (36)
	2		n.o.	n.o.	467 (<1)	294 (<1)	266 (100)	163 (15)	162 (71)	135 (14)	n.o.	69 (14)	58 (31)
MDA	1	472	472 (5)	n.o.	453 (1)	294 (8)	266 (100)	163 (12)	162 (75)	135 (15)	n.o.	69 (12)	n.m.
	2		472 (2)	n.o.	453 (<1)	294 (8)	266 (100)	163 (12)	162 (71)	135 (15)	n.o.	69 (11)	n.m.
HMMA	1	560	560 (1)	n.o.	n.o.	294 (3)	266 (100)	237 (23)	236 (90)	209 (14)	73 (15)	69 (10)	58 (37)
	2		n.o.	545 (<1)	n.o.	294 (3)	266 (93)	237 (24)	236 (100)	209 (14)	73 (14)	69 (10)	58 (31)
HMA	1	546	546 (14)	531 (<1)	n.o.	294 (5)	266 (81)	237 (22)	236 (100)	209 (7)	73 (17)	69 (8)	n.m.
	2		546 (13)	531 (1)	n.o.	294 (6)	266 (87)	237 (23)	236 (100)	209 (7)	73 (18)	69 (9)	n.m.
MDAOH	1	560	n.o.	545 (8)	n.o.	294 (1)	266 (100)	163 (17)	162 (37)	135 (22)	73 (8)	69 (8)	n.o.
	2		n.o.	545 (6)	n.o.	294 (<1)	266 (100)	163 (19)	162 (67)	135 (26)	73 (2)	69 (9)	n.o.

[a] Peaks were considered to be of significant importance if the signal-to-noise ratio was >3.
[b] *N*-HFBP-*O*-TMS = *N*-heptafluorobutyryl-(*S*)-prolyl-*O*-trimethylsilyl; MDMA = 3,4-methylenedioxymethamphetamine; MDA = 3,4-methylenedioxyamphetamine; HMMA = 4-hydroxy-3-methoxymethamphetamine; HMA = 4-hydroxy-3-methoxyamphetamine.
[c] Sequence based on consecutive retention times.
[d] n.o. = Not observed; n.m. = not measured, because outside the selected mass range.

observed especially for the *N*-*O*-TMS derivative of MDAOH. Also for the *O*-TMS derivatives of one enantiomer of HMMA and both enantiomers of HMA, the $[M - CH_3]^+$ fragment was observed, although at very low abundance. The $CH_3^{\cdot}$ radical probably originates from the TMS moiety; this fragmentation is very general, occurring during EI of TMS ethers and esters.[24]

In Table 2, mass spectrometric data are presented for the PCI mode. Although in all spectra the MH^+ ions were found to be the base peak, the characteristic EI fragments a_1 and a_2 were also prominent. The main difference between the spectra of the two ionization modes is that, compared with fragment a_2, the presence of fragment a_1 is more pronounced in the PCI mode than it is in the EI mode. Such subtle differences in the relative abundances of ions which differ by only one atomic mass unit and are formed by different mechanisms have, for example, also been observed between the ions at *m/z* 70 and 71 in the EI and PCI mass spectra of *N*,*N*′-dimethylpiperazine.[25]

Characteristic for the PCI mode is the formation of fragment *c* and of the fragments d_1–d_8 (Table 2). Especially the *d* fragments are of interest because they form characteristic ion clusters, easily recognized in the spectra. The formation of the series of *d* fragments probably includes a cleavage of the prolyl ring followed by a rearrangement. Selective deuterium and oxygen labeling combined with tandem mass spectrometry and/or high-resolution mass spectrometry should give a more detailed understanding of the fragmentation and mechanism.

Increased fragmentation in the ion trap when using the PCI mode has been reported by Boswell *et al.*[26] and may be caused by the relatively long residence time of the ions, by which the chances of collisions are increased. However, collisional stabilization of ions by the helium buffer gas has an adverse effect and may compete with the overall increased fragmentation. As discussed by Boswell *et al.*,[26] different ionization mechanisms operate under PCI conditions in the ion trap, including proton transfer giving rise to protonated molecules and charge transfer resulting in EI-like molecular ions. With respect to the latter process, transfer of electrons from the sample molecule to reactant gas ions takes place, resulting in odd-electron molecular ions, which may fragment further by radical-induced fragmentation. The PCI mode has the advantages that it provides high-mass information and that some fragmentation remains, allowing elucidation of structural details, making it the ionization mode of choice for the purposes of the present study.

Enantiomeric ratios of MDMA and metabolites in urine

The selected ion chromatograms of the GC/MS analysis of the urine sample obtained from an MDMA intoxicated person are shown in Fig. 3. Peak identification of MDMA, MDA, MDAOH, HMMA and HMA was based on both the mass spectrum and the relative retention time. In all three samples MDMA and MDA were identified. In one sample, both HMMA and HMA were found, in the second one only a trace of HMMA and in the third no additional metabolites at all. The presence of these metabolites in human urine is in agreement with earlier reports.[10–12] In two of the samples studied, also the presence of the enantiomers of *N*-hydroxy-(*RS*)-3,4-methylenedioxyamphetamine (MDAOH; Fig. 1) was observed at retention times of 22.43 and 24.03 min, respectively (mass spectra, Fig. 4(A) and (B)).

Based on the peak heights of the protonated molecule, the absolute or 'analytical' enantiomeric ratio of the reference standards was found to be >1 for MDMA

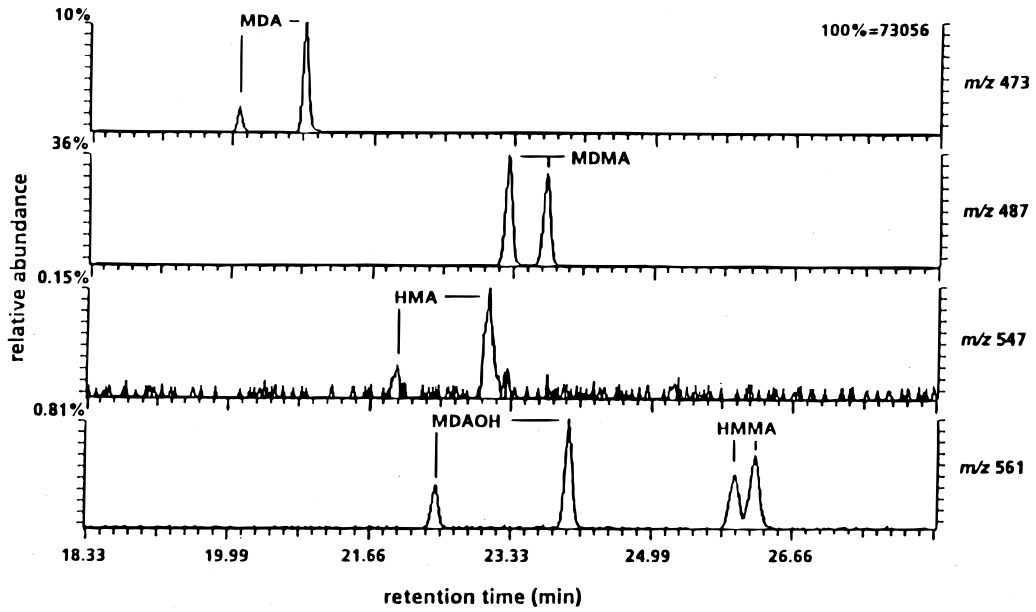


Figure 3. Selected ion chromatograms of the protonated molecules (see Table 2) of the *N*-(*S*)-HFBP-*O*-TMS derivatives of MDMA, MDA, MDAOH, HMMA and HMA, as observed with positive chemical ionization GC/MS after the isolation of the compounds from urine of an intoxicated person, and derivatization with *N*-heptafluorobutyryl-(*S*)-prolyl chloride and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. MDMA = 3,4-methylenedioxymethamphetamine; MDA = 3,4-methylenedioxyamphetamine; MDAOH = *N*-hydroxy-3,4-methylenedioxyamphetamine; HMMA = 4-hydroxy-3-methoxymethamphetamine; HMA = 4-hydroxy-3-methoxyamphetamine.

Table 2. Partial mass spectrometric data[a] for *N*-HFBP-*O*-TMS derivatives of MDMA and its chiral metabolites using the positive chemical ionization mode

			m/z values of characteristic fragments (normalized on the base peak)[d]										
Compound[b]	Enantiomer No.[c]	M_r	MH^+	$[M-CH_3]^+$	*c*	$C_3F_7CONC_4H_7^+$	d_1/d_2	d_3/d_4	d_5	d_6/d_7	d_8	a_1	a_2
MDMA	1	486	487 (100)	n.o.	365 (2)	266 (2)	234 (<1)	232 (3)	205 (8)	203 (8)	201 (15)	163 (64)	162 (24)
	2		487 (100)	n.o.	365 (2)	266 (2)	234 (<1)	232 (3)	205 (8)	203 (9)	201 (7)	163 (78)	162 (28)
MDA	1	472	473 (100)	n.o.	351 (2)	266 (8)	220 (4)	218 (4)	205 (11)	203 (7)	201 (15)	163 (24)	162 (26)
	2		473 (100)	n.o.	351 (2)	266 (4)	220 (3)	218 (3)	205 (8)	203 (6)	201 (11)	163 (20)	162 (24)
HMMA	1	560	561 (100)	n.o.	365 (7)	266 (<1)	n.o.	306 (3)	279 (7)	277 (22)	275 (29)	237 (56)	236 (38)
	2		561 (100)	n.o.	365 (9)	266 (<1)	n.o.	306 (3)	279 (5)	277 (26)	275 (31)	237 (69)	236 (43)
HMA	1	546	547 (100)	n.o.	351 (1)	266 (1)	294 (4)	292 (1)	279 (2)	277 (4)	275 (8)	237 (5)	236 (8)
	2		547 (100)	n.o.	351 (1)	266 (1)	294 (4)	292 (1)	279 (2)	277 (4)	275 (8)	237 (6)	236 (9)
MDAOH	1	560	561 (100)	545 (18)	439 (4)	266 (15)	n.o.	n.o.	205 (8)	203 (11)	201 (11)	163 (20)	162 (24)
	2		561 (100)	545 (12)	439 (3)	266 (7)	n.o.	n.o.	205 (9)	203 (16)	201 (13)	163 (26)	162 (27)

[a–d] See Table 1.

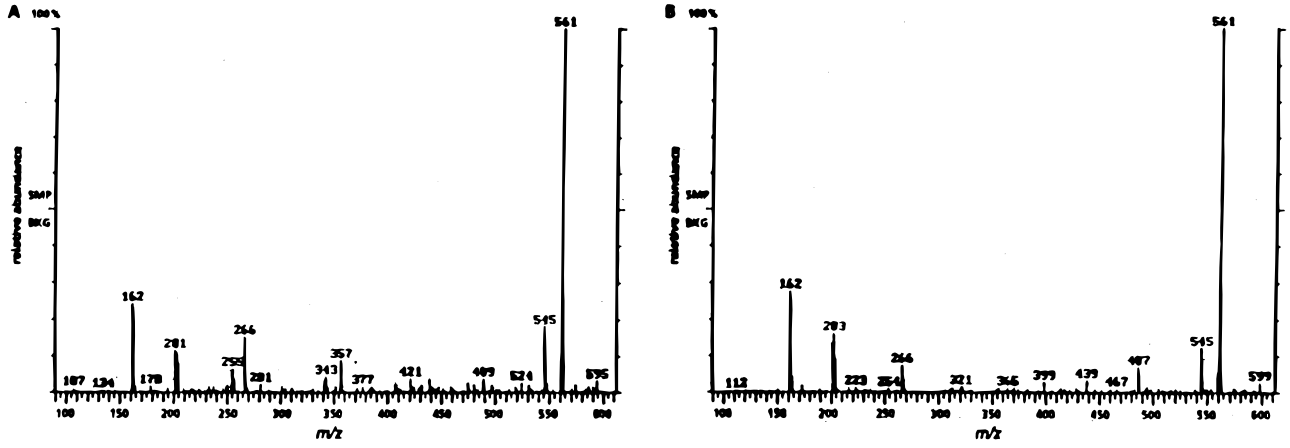


Figure 4. Positive chemical ionization mass spectra of the derivatives of two enantiomers of *N*-hydroxy-3,4-methylenedioxyamphetamine at the retention times of (A) 22.43 and (B) 24.03 min (see also Fig. 3).

and MDAOH and <1 for the other reference standards. The observation that an 'analytical' enantiomeric ratio is not equal to 1 is not uncommon; it can be caused by a difference in instrumental response and/or derivatization efficiency of the individual enantiomers.[23] The 'metabolic' enantiomeric ratios, normalized to the 'analytical' ratio, of all *N*-demethylated metabolites were significantly different from the 'analytical' ratios (Table 3). The 'metabolic' enantiomeric ratios of MDMA and HMMA were >0.5 and those of the *N*-demethylated metabolites MDA, MDAOH and HMA were <0.5. These preliminary results of the analysis of urine samples suggest an enantioselective metabolism for MDMA in humans. This would be in agreement with studies in mice and in rats,[2] where the enantiomeric profiles of the *N*-demethylated metabolites MDA and HMA (*R*/*S* ratio <1) are the reverse of those of MDMA and HMMA (*R*/*S* ratio >1) in 24 h pooled urine. In our study, we used the racemic mixtures as reference material. Therefore, we could not assign the (*R*)- or (*S*)-conformation to the individual enantiomers of the compounds.

N-Hydroxylated chiral metabolites

N-Hydroxylated metabolites of amphetamine-like compounds in human urine have been reported for phentermine and chlorphentermine[27] and were suggested to be intermediates in the metabolic route leading to hippuric acid conjugates (Fig. 5). However, the presence of MDAOH in the urine samples studied was a surprise as, even if formed, *N*-hydroxylated compounds in general[28] and MDAOH itself[29] are known to be relatively unstable. In our study MDAOH was only observed after enzymatic hydrolysis, which means that it was present in urine as a conjugate with either glucuronic acid or sulfate. MDAOH may therefore be more stable in a conjugated form than when unconjugated.

Because MDAOH itself is sometimes the main pharmacologically active compound in illicitly produced 'Ecstasy' tablets, it cannot be excluded that the presence of MDAOH is caused by the combined use of MDMA and MDAOH tablets, especially since up to now metabolic studies of 3,4-methylenedioxyamphetamine-like

Table 3. Absolute and normalized ratios of the enantiomers of MDMA and its chiral metabolites[a]

Compound[b]	Mean of reference standards ($n = 5$) Absolute	Sample 1 Absolute	Sample 1 Normalized	Sample 2[c] Absolute	Sample 2[c] Normalized	Sample 3[c] Absolute	Sample 3[c] Normalized
MDMA	1.12 ± 0.09	1.21	1.08	1.19	1.06	1.27[f]	1.14
MDA	0.61 ± 0.03	0.23[d]	0.38	0.33[d]	0.54	0.28[d]	0.46
HMMA	0.85 ± 0.10	0.74[e]	0.87	n.q.	—	n.q.	—
HMA	0.67 ± 0.07	0.29[d]	0.43	n.o.	—	n.o.	—
MDAOH	1.47 ± 0.13	0.40[d]	0.27	n.o.	—	0.45[d]	0.31

[a] First peak/second peak height ratios were normalized on the absolute ratio of the respective reference standards, which were considered to contain equal amounts of both enantiomers.
[b] *N*-HFBP-*O*-TMS = *N*-heptafluorobutyryl-(*S*)-prolyl-*O*-trimethylsilyl; MDMA = 3,4-methylenedioxymethamphetamine; MDA = 3,4-methylenedioxyamphetamine; HMMA = 4-hydroxy-3-methoxymethamphetamine; HMA = 4-hydroxy-3-methoxyamphetamine; MDAOH = *N*-hydroxy-3,4-methylenedioxyamphetamine.
[c] n.o. = Not observed; n.q. = present, but impossible to quantify correctly.
[d] $p < 0.005$.
[e] $p < 0.1$.
[f] $p < 0.05$.

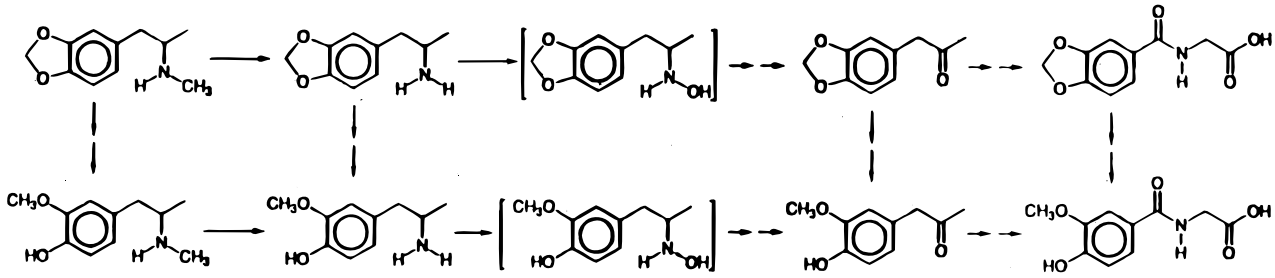


Figure 5. Assumed metabolism of 3,4-methylenedioxymethamphetamine in humans.[12]

compounds did not report the presence of MDAOH in urine.[10–12,30] However, the low frequency of the presence of MDAOH in 'Ecstasy' tablets[8] and the fact that MDAOH was found in two out of three urine samples from totally different and unrelated intoxications suggest that the observed MDAOH is a metabolite rather than a co-administered compound. The fact that the'metabolic' enantiomeric ratios of MDAOH were in the same range as those of MDA (Table 3) also suggests that MDA is the precursor (Fig. 5) of MDAOH in these two cases. The significance of this observation is that MDAOH is converted in the body into 3,4-methylenedioxyphenylacetone (Fig. 5), probably via the intermediate 3,4-methylenedioxy-2-nitroso-1-phenylpropane.[31] Since nitroso compounds are known to be relatively toxic, a sudden accumulation of 3,4-methylenedioxy-2-nitroso-1-phenylpropane, for several possible metabolic reasons, could contribute to acute MDMA intoxications.

Unambiguous identification or exclusion of MDAOH as a metabolite of MDMA in biological samples from MDMA excretion experiments with human subjects and from intoxicated persons is therefore of special interest. It would not only be important for the toxicological aspects of MDMA and other 3,4-methylenedioxyamphetamine-like compounds, but also for their pharmacodynamics, since MDAOH is known to be pharmacologically active.[21] This would mean that the overall pharmacological effect of MDMA is determined by three compounds, since the parent compound and the metabolite MDA are also pharmacologically active. Further research will be performed in order to fit the developed methodology for quantitative purposes as for typical pharmacokinetic experiments and more extensive toxicological studies.

The presence of the minor chiral metabolites 3,4-dihydroxymethamphetamine and 3,4-dihydroxyamphetamine[11,12] was not observed. However, because the compounds were not available as reference compounds in our laboratory, the isolation procedure used in this study could not be validated for these compounds. In principle, our GC/MS assay and the derivatization method should also be applicable to detect the dihydroxy metabolites.

CONCLUSIONS

A chiral assay for MDMA and its major metabolites has been selected and developed. The procedure was based on the *N*-(*S*)-HFBP-*O*-TMS derivatization method and GC/MS analysis in the PCI mode. Preliminary results of the analysis of urine samples showed the usefulness of the assay and indicated an enantioselective metabolism in the *N*-demethylation pathway for MDMA in humans.

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